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Epidemiological Study and Genetic Analysis of GB Virus C Infection in General Population From an Area Endemic for Hepatitis C

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The aim of this work was to study the prevalence, potential risk factors, clinical and laboratory features of GB virus C (GBV-C) infection in general population from an area endemic for hepatitis C. A reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of GBV-C RNA was used to examine the prevalence of GBV-C RNA in both hepatitis C virus (HCV) endemic (R town) and nonendemic areas (M town) in Yamagata prefecture, Japan. In R town, GBV-C RNA was detected in 23 (2.9%) out of the 800 residents, whereas anti-HCV and HCV-RNA were found in 226 (28.3%) and 163 (20.4%), respectively. The prevalence of GBV-C RNA in R town (2.9%) was higher than that in M town (1.0%), although the difference was not statistically significant. The individuals with anti-HCV had significantly higher frequency of active GBV-C infection than those without anti-HCV in both towns. No evidence indicating that GBV-C infection affected the severity of hepatitis C was obtained. The multivariate analysis revealed that the young anti-HCV positive individuals with a history of blood transfusion had higher incidence of active GBV-C infection. The phylogenetic analysis showed that the GBV-C isolates from both R and M towns were divided into two separate branch groups designated HG and Asia GB groups. ***J. Med. Virol. 54:237–242, 1998.***
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KEY WORDS: GBV-C; HGV; HCV; epidemiology; phylogenetic tree

INTRODUCTION

Since the discovery of hepatitis C virus (HCV) and hepatitis E virus (HEV), the search for other so-called non-A-E viral hepatitis agents has been intense. Recently, identification of the GB virus C (GBV-C) [Simons et al., 1995] and the hepatitis G virus (HGV) [Linnen et al., 1996] opened a field for studying the association of human liver diseases with these new agents. Both GBV-C and HGV are positive-stranded RNA viruses and members of the *Flaviviridae* family. Sequence analysis revealed that GBV-C and HGV are virtually identical (approximately 95% amino acid identity) [Shao et al., 1996], suggesting that they are independent isolates of the same virus. Therefore, we referred to this virus as GBV-C.

The fact that GBV-C was originally isolated from the sera of some cryptogenic hepatitis patients [Simons et al., 1995] and the reports of fulminant hepatitis associated with GBV-C infection [Yoshiba et al., 1995; Heringlake et al., 1996] implied that GBV-C may be a novel causative agent of human hepatitis. However, further studies on GBV-C infection in patients on hemodialysis maintenance [Masuko et al., 1996] and in organ recipients [Berenguer et al., 1996; Fried et al., 1997] indicated that GBV-C may represent a mild infection without causing significant liver injury. More recently, investigations on the role of GBV-C infection in community-acquired acute hepatitis [Alter et al., 1997] and posttransfusion hepatitis [Alter et al., 1997a] provided a strong evidence that most GBV-C infections are not associated with hepatitis. Although the clinical significance of GBV-C infection still remains unclear, there is no doubt that GBV-C results in acute and persistent infection in humans [Alter et al., 1997], and that the virus is parenterally transmitted [Linnen et al., 1996; Alter et al., 1997a]. The prevalence of GBV-C infection was evaluated in various groups that included volunteer blood donors and recipients [Linnen et al., 1996; Alter et al., 1997a], organ recipients [Berenguer et al., 1996; Fried et al., 1997; Murthy et al., 1997], intravenous drug users (IVDUs) [Linnen et al., 1996; Wu et al., 1997], and patients with

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TABLE I. Characteristics of Selected Residents From the M and R Towns

Characteristics, number (%)	M town (n = 200)	R town (n = 800)	*P*
Age[a]	62.6 ± 10.9	61.5 ± 10.5	0.175[c]
Male sex	74 (37.0)	351 (43.9)	0.079[d]
HBsAg	8 (4.0)	16 (2.0)	0.098[d]
Anti HCV	7 (3.5)	226 (28.3)	0.001[d]
HCV-RNA	5 (2.5)	163 (20.4)	0.001[d]
Alanine aminotransferase level (IU/liter)[a,b]	20.5 ± 10.9	22.9 ± 21.0	0.029[c]
Blood transfusion	10 (5.0)	111 (13.9)	0.001[d]
Operation	70 (35.0)	306 (38.3)	0.396[d]
Acupuncture	51 (25.5)	183 (22.9)	0.433[d]
Alcohol abuse	52 (26.0)	198 (24.8)	0.715[d]

[a]Plus–minus values are mean ± SD.
[b]Alanine aminotransferase data was missing for two residents in R town.
[c]t-test.
[d]Fisher's exact test.

chronic viral hepatitis [Linnen et al., 1996; Tanaka et al., 1996; Zhang et al., 1997]. GBV-C RNA was found in 1–4.2% of volunteer blood donors, approximately 20% of IVDUs and 10–20% of patients with chronic hepatitis C [Alter, 1997b]. Therefore, it can be inferred that the general population at risk for exposure to HCV may also be at risk for infection with GBV-C. To our knowledge, the prevalence of GBV-C RNA in the general population of hepatitis C endemic areas has not been reported so far.

In this study, we investigated the prevalence, risk factors, clinical and laboratory features of GBV-C infection in 800 residents in a hepatitis C endemic area. Two hundred randomly selected residents and 100 anti-HCV positive individuals in a hepatitis C nonendemic area were also analyzed for comparison. Moreover, partial nucleotide sequences of the viral genome from all GBV-C positive individuals were determined and compared with those of GBV-C isolates reported previously from Japan and other countries.

MATERIALS AND METHODS

Subjects

From January 1991 to June 1993, a mass survey of liver function was conducted in 67,810 residents from six towns of Yamagata prefecture, Japan [Ishibashi et al., 1996]. The survey revealed a high prevalence of anti-HCV in the residents of R town (27.8% of 2,915 residents aged 40 or older) and a low prevalence of anti-HCV in those of M town (3.1% of 3,194 residents aged 40 or older). Here, 800 of the previously surveyed 2,915 residents in R town were randomly selected. A control group, composed of 200 randomly selected individuals and 100 anti-HCV positive individuals (including 7 of the 200 randomly selected) from M town, was selected for comparison. Characteristics of the selected residents from M and R towns are shown in Table I. All samples analyzed in this study were collected during the survey described above and stored at −40°C before testing for GBV-C.

Markers for HBV and HCV Infection

HBsAg was assayed using commercially available radioimmunoassay kits (AUSZYME II, Dainabot, Tokyo, Japan). Anti-HCV antibody was measured by commercially available second-generation enzyme-linked immunosorbent kit (Abbott HCV EIA 2.0, Dainabot). HCV RNA was detected by RT nested PCR with 5′ noncoding region-derived primers [Bukh et al., 1992]. The HCV genotype was determined by PCR with genotype-specific primers derived from core region according to the Okamoto method [Okamoto et al., 1992].

Detection of GBV-C RNA

As described previously [Zhang et al., 1997], GBV-C RNA was detected by RT nested PCR with 5′ noncoding region-derived primers. Briefly, GBV-C RNA was extracted from 100-μl serum and reverse-transcribed to cDNA with 100-pmol random primer and 200-u M-MLV reverse transcriptase. The cDNA was subjected to the first round of PCR with TaKaRa Taq DNA polymerase using the outer primers (sense, sw: 5′-GACAGGGTTGGTAGGTCGTAAA-3′; antisense, awm: 5′-CCCACTGGTCCTTGTCAACT-3′). The PCR was performed for 30 cycles (94°C for 15 sec; 55°C, 15 sec; 72°C, 65 sec) in a thermocycler PE 2400. For the second round of PCR, 1 μl of the first PCR product was reamplified as described above by using the inner primers (sense, sn: 5′-GTTGGTAGGTCGTAAATCCG-3′; antisense, an: 5′-AGAGAGACATTGAAGGGCGAC-3′) and a primer annealing temperature of 55°C. The second PCR product was analyzed by electrophoresis on a 2% agarose gel. A number of precautions [Kwok and Higuchi, 1989] were taken to reduce the risk of contamination during the PCR reaction. To verify the specificity of the PCR product, all positive samples were repeatedly analyzed by two different individuals.

Sequence and Molecular Evolutionary Analysis

The amplified DNA was purified with QIA quick PCR purification kits (QIAGEN, Hilden, Germany), and directly sequenced by fluorescent dye terminator cycle sequencing method using an ABI DNA sequencer 373A (Applied Biosystems, Foster City, CA). The evolutionary relationship between the GBV-C positive samples and the previously reported GBV-C strains was elucidated by the six-parameter method [Gojobori et al., 1982]. The phylogenetic tree was constructed by the unweighted pair-group method with arithmetic mean (UPGMA) in ODEN program (version 1.1) [Ina, 1994]. The robustness of the phylogenetic tree was assessed using bootstrap method [Felsenstein, 1985] based on 100 replicates.

Statistical Analysis

Categorical variables were compared using a Fisher's exact test or chi-square test, and continuous variables were compared using a Student's t-test. Multivariate logistic regression model was applied to assess potential confounding factors on GBV-C infection. All

TABLE II. Prevalence of GBV-C RNA and Anti-HCV in 800 Residents From R Town

Age groups (years)	GBV-C RNA positive/total (%)				Anti-HCV positive/total (%)			
	Males	Females	Total	*P*	Males	Females	Total	*P*
40–49	5/69 (7.3)	1/68 (1.5)	6/137 (4.4)		11/69 (15.9)	12/68 (17.7)	23/137 (16.8)	
50–59	0/67 (0)	5/115 (4.4)	5/182 (2.8)		18/67 (26.9)	28/115 (24.4)	46/182 (25.3)	
60–69	1/131 (0.8)	5/169 (3.0)	6/300 (2.0)		41/131 (31.3)	61/169 (36.1)	102/300 (34.0)	
70–79	3/72 (4.2)	3/86 (3.5)	6/158 (3.8)		19/72 (26.4)	32/86 (37.2)	51/158 (32.3)	
⩾80	0/12 (0)	0/11 (0)	0/23 (0)	0.540[a]	0/12 (0)	4/11 (36.4)	4/23 (17.4)	0.002[a]
Total	9/351 (2.6)	14/449 (3.1)	23/800 (2.9)	0.642	89/351 (25.4)	137/449 (30.5)	226/800 (28.3)	0.108

[a]Chi-square test for 2 × 5 contingency table, otherwise, for 2 × 2 contingency table.

tests were two-sided and a significance level of 0.05 was used for all comparisons.

RESULTS

Out of 800 residents in R town, GBV-C RNA was detected in 23 (2.9%) individuals, whereas anti-HCV and HCV-RNA were found in 226 (28.3%) and 163 (20.4%) individuals, respectively. The prevalence of GBV-C RNA in R town (2.9%) was higher than that in M town (1.0%); however, the difference was not statistically significant ($P = 0.202$).

There were no significant differences in prevalence of GBV-C RNA across the gender groups and the age groups for the 800 residents from R town. Anti-HCV did not appear to be associated with gender; however, the prevalence of anti-HCV was significantly different among age groups ($P = 0.002$) (Table II). Notice that the frequency of active GBV-C infection was significantly higher in the individuals with anti-HCV (8.4%, 19/226) than those without anti-HCV (0.7%, 4/574; $P < 0.001$). Because the number of anti-HCV positive subjects from 200 randomly selected in M town was insufficient to acquire a meaningful prevalence, sera was collected from an additional 100 anti-HCV positive individuals residing in this town. The prevalence of GBV-C RNA in the subjects seropositive for HCV (5%, 5/100) was also significantly higher than for those seronegative in M town (0.5%, 1/193; $P = 0.019$), which suggests a positive association of active GBV-C infection with exposure to HCV infection.

Clinical and epidemiological features of the subjects with and without GBV-C RNA from R town are summarized in Table III. Among the 23 individuals with GBV-C infection, 19 were positive for anti-HCV. The mean ALT level (17.3 ± 10.0 u/L) of four individuals infected with GBV-C alone was similar to that of the 570 residents infected with neither GBV-C nor HCV (18.7 ± 11.7 u/L), but significantly lower than that of the 207 individuals infected with HCV alone (33.1 ± 33.3 u/L). The mean ALT level of the 19 individuals with both GBV-C and HCV infections (38.4 ± 24.2 u/L) was comparable to that of the individuals infected with HCV alone (33.1 ± 33.3 u/L). There was also no significant difference in clinical features, such as jaundice and symptoms (data not shown), between the individuals with HCV infection alone and those with HCV and GBV-C co-infection.

All potential risk factors (age, male sex, HBsAg, etc.) listed in Table III are evaluated by univariate and multivariate methods for their relationship to GBV-C infection. Results of the univariate analysis suggested that anti-HCV, HCV-RNA, and history of blood transfusion were each significantly related to GBV-C infection; individuals with anti-HCV, HCV-RNA, and a history of blood transfusion showed a higher incidence of active GBV-C infection. The multivariate analysis concluded that the variables most significantly related to GBV-C infection were anti-HCV, history of blood transfusion, and age. On the basis of the regression coefficients, the probability of GBV-C RNA positive in R town can be estimated by substituting values of the risk factors into the regression equation:

$$\log \frac{P}{1-P} = -6.7373 + 2.3923\ (\text{anti–HCV}) + 1.2096\ (\text{blood transfusion}) - 0.0340\ (\text{age}).$$

Obviously, the young anti-HCV positive individuals with a history of blood transfusion displayed a higher incidence of active GBV-C infection.

The nucleotide sequence identity of the 5′ noncoding region among the isolates ranged from 90% to 100%, irrespective of the town from which the isolates were obtained. The phylogenetic tree was constructed in order to examine the genetic relationship between our GBV-C isolates and the previously reported GBV-C strains. As shown in Figure 1, the isolates that were examined could be divided into three distinct branch groups. The isolates from R as well as M town clustered into two branches, one containing the prototype of HGV strains (HG group), the other tentatively named as Asia GB group. Bootstrap analysis showed that the GBV-C isolates belonging to the HG and Asia GB groups form clusters with replicate values of 98% and 96%, respectively.

DISCUSSION

GBV-C is a new member of the *Flaviviridae,* and its epidemiology and clinical significance have not yet been defined. However, high frequency of GBV-C and HCV coinfection has been observed in hepatitis patients and persons with risks of parenteral hepatitis virus infection [Alter, 1997b], which suggest that GBV-C and HCV perhaps share common transmission routes. Hence, it is likely that the general population in

TABLE III. Characteristics of 800 Residents in R Town According to Their GBV-C and HCV Infection Status

Characteristics, number (%)	GBV-C alone (n = 4) A	HCV alone (n = 207) B	GBV-C and HCV (n = 19) C	Non GBV-C and HCV (n = 570) D	*P*	Multiple comparison[e]
Age						
<65	3 (75)	106 (51)	12 (63)	343 (60)	0.001[d]	B&D is significantly different
⩾65	1 (25)	101 (49)	7 (37)	227 (40)	0.123[d]	
Year[a]	56.3 ± 14.9	63.6 ± 8.8	61.0 ± 10.1	60.8 ± 11.0	0.009[c]	B&D is significantly different
Male sex	3 (75)	83 (40)	6 (32)	259 (45)	0.220[d]	
Alanine aminotransferase level, IU/liter[a,b]	17.3 ± 10.0	33.1 ± 33.3	38.4 ± 24.2	18.7 ± 11.7	<0.001[c]	B&D, C&D are significantly different
HBsAg				16 (3)		
HCV-RNA		148 (71)	15 (79)		0.600[d]	
HCV genotype						
1b		75 (36)	11 (58)		0.083[d]	
2a		6 (3)	0 (0)		1.000[d]	
2b		65 (31)	3 (16)		0.197[d]	
mixed		2 (1)	1 (5)		0.233[d]	
Blood transfusion	2 (50)	53 (26)	9 (47)	47 (8)	<0.001[d]	A&D, B&D, C&D are significantly different
Operation	1 (25)	104 (50)	12 (63)	189 (33)	<0.001[d]	B&D, C&D are significantly different
Acupuncture	1 (25)	66 (32)	7 (37)	109 (19)	0.001[d]	B&D is significantly different
Alcohol abuse	2 (50)	37 (18)	2 (11)	157 (28)	0.007[d]	B&D is significantly different

[a]Plus–minus values are mean ± SD.
[b]Alanine aminotransferase data was missing for one with HCV alone, and one with non GBV-C and HCV.
[c]t-test.
[d]Fisher's exact test.
[e]Fisher's LSD test.

an area where HCV infection is highly prevalent might exhibit a higher incidence of GBV-C infection. In order to clarify this point, we investigated the prevalence of GBV-C RNA in both hepatitis C endemic (R town) and nonendemic (M town) areas based on detection of GBV-C RNA by RT-PCR. It was found that the prevalence rates of GBV-C RNA were 2.9% and 1.0% in R and M towns, respectively. This finding was rather unexpected, since 28.3% and 20.4% of the randomly selected residents in R town were positive for anti-HCV and HCV-RNA, respectively, suggesting that the prevalence of GBV-C RNA was much lower than that of HCV-RNA in HCV endemic area. It raised the issue whether our results have underestimated the prevalence of GBV-C RNA due to the technical limitation of RT-PCR. The GBV-C RNA detection performed here was based on a highly sensitive and reproducible RT nested PCR as demonstrated by multiple primer sets comparison [Zhang et al., 1997]. Nevertheless, it is impossible to exclude the possibility that our technique detected only a part of the subjects exposed to GBV-C, since PCR may only detect active infection. Recently, Alter et al. [1997a] reported that although GBV-C infection was generally persistent, clearance of the virus occurred in approximately one third of the patients with GBV-C infection within three years after being infected. Tacke et al. [1997] also reported that 9% of 80 healthy donors were positive for anti-E2 (a GBV-C-specific antibody), but all of them were negative for GBV-C RNA. Therefore, using a combination of GBV-C RNA and E2 antibody to estimate the exposure rate as well as the active infection rate in such population is required in future research.

Although the prevalence of GBV-C RNA was considerably lower than that of HCV-RNA in HCV endemic area, the positive rate of GBV-C RNA was significantly higher in the individuals with anti-HCV than those without anti-HCV in both R and M towns. This confirmed previous observations that GBV-C infection could be detected frequently in patients with HCV infection [Alter, 1997b; Zhang et al., 1997]. The association of GBV-C with HCV can be explained if two viruses share the same transmission routes. There are several lines of evidence [Yoshiba et al., 1995; Alter et al., 1997a; Murthy et al., 1997; Wu et al., 1997] that GBV-C can be transmitted by blood transfusion and other parenteral routes, including intravenous drug use. The possible association between GBV-C and several risk factors was evaluated by univariate and multivariate analyses for the subjects in R town, where drug abuse was rare, but where alcohol abuse and acupuncture were common. The variables significantly related to GBV-C infection were found to be anti-HCV positivity, history of blood transfusion, and age in multivariate analysis. It is noteworthy that these results are not entirely consistent with those in the univariate analysis. For instance, age was found to be unrelated to GBV-C infection in the latter analysis. If age was treated as ordinal data and categorized into the subsets as 40–60 and 65–high, or 40–49, 50–59, 60–69, 70–79, and 80–high, it was not rated as a significant factor. If,

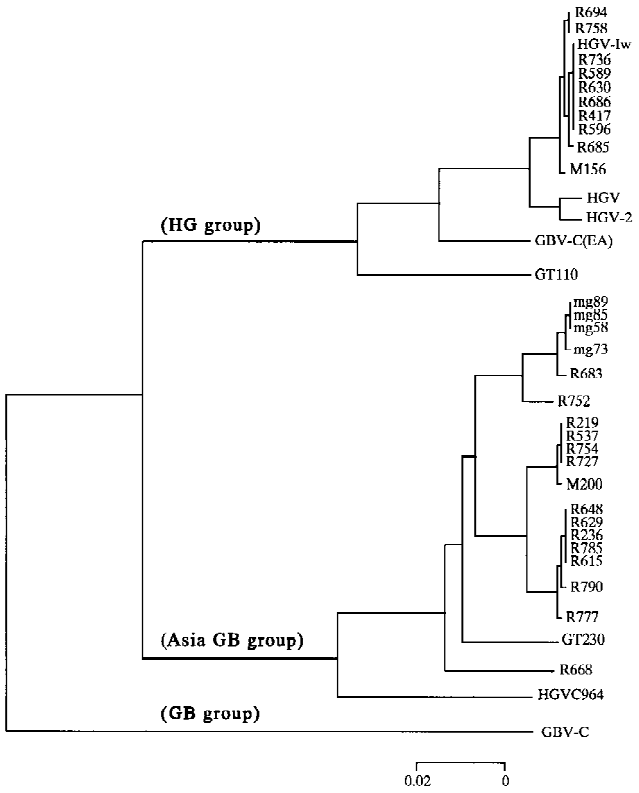

Fig. 1. A phylogenetic tree of 29 GBV-C isolates from R and M town, as well as eight previously reported GBV-C isolates. R denotes the isolates from R town and M/mg indicates the isolates from M town. The nucleotide sequence data reported in this study will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB005489–AB005516. The accession numbers of eight reported isolates are GBV-C, U36380; GBV-C(EA), U63715; HGV, U44402; HGV-2, U45966, HGV-Iw, D87255; HGVC964, U75356; GT110, D90600; and GT230, D90601.

however, age is to be taken as a continuous variable, then it is a risk factor that significantly affects GBV-C.

Another study [Alter et al., 1997] also reported that a young population was more likely to be GBV-C-infected, which was consistent with our results obtained from the multivariate analysis. In addition, the correlation of active GBV-C infection with HCV in younger but not in older persons is consistent with the fact that GBV-C RNA will disappear, whereas HCV persists. It should also be noted that among the 23 individuals infected with GBV-C, there were 8 persons who did not have histories of blood transfusion, operation, acupuncture, and drug abuse, which imply that GBV-C may be transmitted by other routes.

Recently, Stark K., et al. [1996], reported that sexual contact may be an important route of GBV-C transmission. However, among six couples in current study, no intraspousal transmission of GBV-C was observed (data not shown). The substantial epidemiological data relevant to sexual behavior was unavailable in this study, therefore no conclusion was drawn about sexual transmission of GBV-C infection. Although the precise routes of GBV-C transmission are still uncertain, our data indicate that relatively young individuals with anti-HCV and a history of blood transfusion have a higher incidence of active GBV-C infection.

To examine the significance of GBV-C in coinfection with HCV, we compared the clinical and laboratory features among four groups that were classified according to the presence or absence of GBV-C and HCV infections. No evidence was obtained which suggests that GBV-C infection affected the severity of hepatitis C. Mean ALT level was similar between individuals with HCV infection alone and those with combined GBV-C and HCV infection. There was also no significant difference in clinical manifestations between HCV infection alone and HCV/GBV-C coinfection. These results are in agreement with recently published reports [Tanaka et al., 1996; Bralet et al., 1997], which claimed that GBV-C infection had no apparent influence on the clinical course of HCV infection.

Genomic heterogeneity of GBV-C has been documented among different isolates from around the world [Shao et al., 1996; Muerhoff et al., 1997]. Updated phylogenetic analysis [Muerhoff et al., 1997; Mukaide et al., 1997] demonstrate that GBV-C exists as three distinct "genotypes": GB type, HG type, and Asia GB type. However, the divergence in entire genomic sequence among these genotypes, ranging from 12.3% to 13.8% [Shao et al., 1996], was small compared with that observed among the different HCV genotypes (21.5–29%) [Smith et al., 1997]. For this reason, we used the term "group" rather than "genotype" to describe the phylogenetic groups of GBV-C. Our phylogenetic tree showed that the isolates obtained from both R and M towns cluster into two separate branches, HG and Asia GB groups, with none belonging to the GB group that is mainly composed of African isolates represented by the prototype of GBV-C. It should be noted that although the position of GBV-EA in our phylogenetic tree is not the same as that generated from an alignment of 8 full-length genomes, our phylogenetic analysis of 37 GBV-C 5′ NC sequences of 185 nt in length (including GBV-EA) demonstrated the same grouping as those determined from the full-length genomes analysis, in which GBV-EA also belongs to the HG group. The segregation of HG and Asia GB groups here were supported by bootstrap values of 98% and 96%, respectively.

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